Human Peripheral Blood Lymphocytes as a Cell Model to Evaluate the Genotoxic Effect of Coal Tar Treatment

Sofia Pavanello and Angelo Gino Levis

Department of Biology, University of Padova, Padova, Italy

Peripheral blood lymphocytes (PBL) from psoriatic patients therapeutically exposed to polycyclic aromatic hydrocarbons (PAH) during coal tar (CT) treatment were used to evaluate the *in vivo* formation of benzo[*a*]pyrene diol epoxide(BaPDE)-DNA adducts by an ELISA technique and by the ^{32}P-postlabeling method. Moreover, we controlled if the pretreatment with CT influences the formation of BaP-DNA adducts and the BaP metabolism in the PBL obtained from psoriatic patients, treated *in vitro* with BaP. Our data did not show any significant influence of the CT treatment on the levels of PAH-DNA adducts. Moreover, the use of PBL from psoriatic patients, treated *in vitro* with BaP, did not allow to detect significant modifications of the metabolic activation of BaP and of the ability of its metabolites to bind to DNA, before and after CT treatment. Thus, PBL do not seem to represent an useful cell model to evaluate the possible genotoxic effect of the exposure through the skin of psoriatic patients to the PAH contained in CT. —Environ Health Perspect 102(Suppl 9):95–99 (1994)

Key words: psoriatic patients, peripheral blood lymphocytes, coal tar, DNA adducts, ELISA, ^{32}P-postlabeling

Introduction

Polycyclic aromatic hydrocarbons (PAH) are widely distributed in the environment and the exposure of humans to PAH is thought to be responsible, at least partly, for the higher lung cancer incidence in smokers than in nonsmokers, and in some groups of workers than in the general population (*1,2*). Epidemiological studies have also indicated an association between human occupational exposure to coal tar (CT), which is generally characterized by an high content of PAH, and skin cancer (*3*). Pharmaceutical grade CT solutions are also employed for therapeutical purposes, e.g., for the treatment of psoriasis, which is a common proliferative skin disease, not accompanied by malignant cell transformation, that affects 2 to 3% of the human population (*4*). The high exposure of psoriatic patients to genotoxic agents is documented by the exceptional levels of PAH metabolites and/or mutagenic compounds excreted in their urine, as well as by the significant levels of chromosomal damages detected in their lymphocytes (*5*).

This article was presented at the IV European ISSX Meeting on Toxicological Evaluation of Chemical Interactions: Relevance of Social, Environmental and Occupational Factors held 3–6 July 1992 in Bologna, Italy.

This work was supported by the Italian Association for Research on Cancer (A.I.R.C.) and it is included in the ACRO project (C.N.R.).

Address correspondence to Dr. Sofia Pavanello, Department of Biology, University of Padova, Via Trieste 75, 35121 Padova, Italy. Telephone 39 49 8286276.

PAH require metabolic activation to exert their carcinogenic activity. In particular, the metabolism of benzo[*a*]pyrene (BaP), which is the most widely studied carcinogenic PAH compounds, is highly stereoselective: over 30 different metabolites have been identified, among which the carcinogenic activity was related to the formation of BaP diol epoxide (BaPDE) intermediates (*6*).

Since the covalent binding of the reactive carcinogenic metabolites with DNA is believed to be the first step in the initiation of the carcinogenic process (*7*), the monitoring of PAH-DNA adducts has been included in studies of human exposures to carcinogens as a very sensitive molecular index providing the quantitative measurements of the biologically effective dose of the environmental carcinogens (*8*).

In experimental animals, DNA adduct levels in target tissues are related to the dose administered to the animal and the ultimate carcinogenic response (*9*). In human studies, target tissue samples can hardly be obtained. The relative ease with which peripheral human blood lymphocytes (PBL) can be collected prompted the use of PBL as a surrogate for the target tissues in order to detect the effects of human exposure to environmental genotoxic agents and to study the *in vitro* metabolism of such agents.

Materials and Methods

Subjects Analyzed

Male psoriatic patients, presenting cutaneous lesions involving 20 to 100% of the body surface, excluding the genital area and hands, were analyzed. The patients were hospitalized in the Dermatology Clinic of the University of Padora (Italy). For each patient data regarding the age, smoking habits, occupational or consistent environmental exposure to PAH, medical history, domiciliary treatments for psoriasis and drugs taken during the coal tar therapy, were collected, the most relevant of which are reported in Table 1. During hospitalization, some of the patients were treated with crude coal tar (CT) either alone or in association with a CT-based paste (TP) containing 50% coal tar, sometimes in association with UV-irradiation, or with a 2% coal tar ointment (TO). Controls were male healthy subjects, 25 to 50 years old, free from chronic illness, none subjected to medical prescription or exposures to ionizing radiation or industrial chemicals at work. Nonsmokers were selected as individuals who had never smoked and smokers those consuming 15 or more cigarettes per day at least three months prior to donating the blood.

Cell and DNA Isolation

Samples of peripheral blood (20–30 ml) were collected by venapuncture in heparinized plastic syringes. The purity of the DNA, isolated from cell lysates by cycles of phenol/chloroform extraction (*10,11*), was checked and quantified by UV spectroscopy (the absorbance ratios of DNA at 260/230 nm and 260/280 nm were usually >2.3 and >1.8, respectively).

Table 1. Age, smoking habits, treatment protocol followed, and method of detection of BAPDE-DNA adducts in control human subjects and in psoriatic patients following therapy with coal tar.

Subject	Age	Smoking[a] habits	Days of treatment	Type of[b] treatment	% Body surface with lesions	Method of detection of BaPDE-DNA adducts
1	51	S	8	TP	75	ELISA
2	25	S	7	TP	80	"
3	37	S	6	CT, TP	80	"
4	56	NS	6	TP	65	"
5	58	NS	13	TP	35	"
6	47	NS	8	TP	80	"
7	NA	NA	6	CT, TP	40	"
8	NA	NA	13	CT, TP	20	"
9	23	S	7	TP	35	"
10	NA	NA	7	CT	60	"
11	24	NS	10	TP	20	"
12	58	NS	8	TP	30	"
13	60	NS	6	TP	70	"
14	16	NS	3	TP	75	"
15	48	S	17	CT, TP	20	"
16	60	S	9	TP	70	"
17	34	S	5	TP	70	"
18	45	S	11	CT, TP	30	"
19	58	NS	6	TP	60	"
20	31	NS	5	TP	60	"
21	52	S	10	TO	80	"
22	42	S	10	TO	80	"
23	40	S	10	TO	80	"
1	65	NS	8	TP	70	^{32}P NucleaseP1/butanol
2	38	S	8	TP	70	"
3	47	NS	8	TP	70	"
4	56	NS	8	TP	100	"
5	35	NS	8	TP	60	"
6	43	NS	8	TP	90	"
7	52	S	8	TP	100	"
8	34	S	8	TP	100	"
9	61	NS	8	TP	80	"
10	25	NS	–	–	–	^{32}P NucleaseP1
11	25	NS	–	–	–	"
12	27	S	–	–	–	"
13	30	S	–	–	–	"
14	30	NS	–	–	–	"
15	30	NS	–	–	–	"
16	25	NS	–	–	–	"
17	50	NS	–	–	–	"
1	49	S	4	TP	30	HPLC
2	50	NS	6	TP	90	"
3	43	S	4	TP	80	"
4	65	NS	4	TP	80	"
5	60	NS	4	TP	80	"
6	56	NS	10	TP	80	"
7	30	NS	4	TP	80	"
8	79	NS	8	TP	80	"
9	27	NS	6	TP	40	"
10	70	S	6	TP	80	"
11	25	S	–	–	–	"
12	26	NS	–	–	–	"
13	30	NS	–	–	–	"
14	32	S	–	–	–	"
15	26	NS	–	–	–	"

[a] S, smoker; NS, non-smoker; NA, data not available. [b] CT, pure coal tar; TP, coal tar based paste; TO, coal tar based ointment; UV, ultraviolet radiation.

Detection of BaPDE-DNA Adducts (ELISA and ^{32}P-postlabeling)

ELISA assays were performed as described elsewhere (*10*) using rabbit anti BaPDE-DNA polyclonal antiserum (F29). Human DNA samples were tested in two to six separate experiments (depending on the amount of DNA available). In addition each sample was assayed in triplicate or quadruplicate within each experiment. Different concentrations of ^{3}H-BaPDE-DNA standard were assayed in each experiment in quadruplicate. All assays were performed blindly by coding test samples.

DNA adducts were also detected by ^{32}P-postlabeling as described elsewhere (*11*). Five µg DNA for each sample were digested for 3 hr and 30 minutes at 37°C with 250 mU micrococcal nuclease and 8m U spleen phosphodiesterase. Samples were further digested for 30 minutes with 2 µg nuclease P1. After the addition of 0.5 mM Tris-base buffer, the digested DNA was labeled with 20 µCi of ^{32}P-ATP, 2.5 units of T4 polynucleotide kinase. After 30 minutes, the reaction was terminated by adding 40 mM potato apyrase. Purification and resolution of ^{32}P-labeled adducts were carried out on polyethyleneimine-cellulose TLC sheets.

The isolation of the DNA adducts was also obtained by twice subsequent butanol extractions of the digested DNA samples, essentially as described by Gupta (*12*).

The chromatograms were visualized by autoradiography at -80°C, using intensifying screens. Adduct levels were determined by excising the marked areas of the chromatograms, the radioactivity of which was measured by Cerencov counting. Relative adduct labeling (RAL) was calculated on the basis of the amount of radioactivity on the chromatograms, the DNA amount, and the specific activity of the [^{32}-P] ATP used for the labeling (*11*). In each experiment a ^{3}H-BaPDE-DNA standard with a known modification level was included (recoveries of 80–100% were obtained).

The formation of BaPDE-DNA adducts in PBL treated *in vitro* for 24hr with the BaP active metabolite, 2µ M (-)-BaP-7,8-dihydrodiol, was determined by quantifying by the HPLC technique the amounts of their hydrolisis products, the BaP-tetrols. To this purpose the (-)-BaP-7,8-dihydrodiol metabolites were extracted twice by adding to the culture medium an equal volume of ethyl acetate, saturated with Tris-HCl 10 mM pH 7.5 buffer, and separated by HPLC using a Resolve C18 column as previously described (*13,14*).

Results and Discussion

Detection of BaPDE-DNA Adducts in PBL from Psoriatic Patients by ELISA

The levels of BaPDE-DNA adducts were determined by an ELISA assay in the PBL of 23 psoriatic patients during CT treatment and 2 to 5 months later (Figure

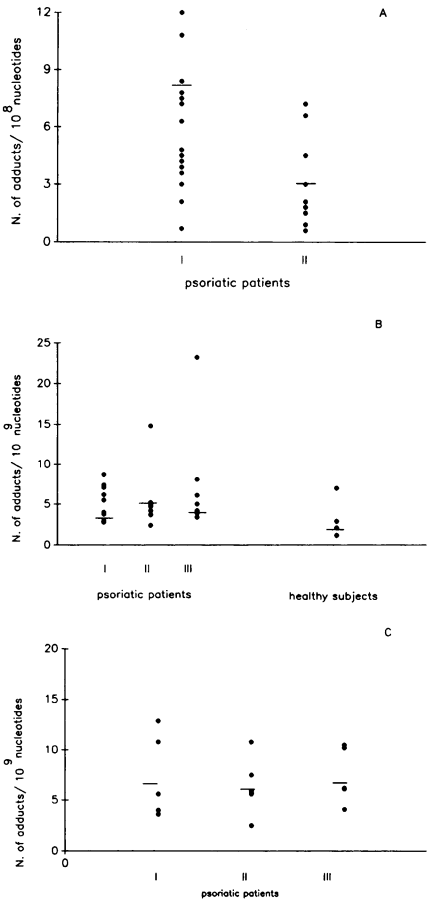

Figure 1. DNA adducts in PBL of psoriatic patients detected by ELISA technique (*A*), and the ^{32}P-postlabeling method using the nuclease P1 (*B*) or the butanol extraction procedure (*C*). Figure 1A: I, during CT treatment; II, 2–5 months after the end of treatment. Figures 1B and 1C: I, before clinical therapy with CT; II, after 8 days of continuous CT application and, III, 16 days after the end of CT therapy.

1A). We found that the mean adduct level during CT treatment was 7.7 ± 4.9 adducts/10^8 nucleotides, a level comparable to those reported for subjects occupationally exposed to PAH. In nine of the 10 patients examined 2 to 5 months after the end of CT therapy, the mean adduct level was significantly lowered down to 3.3 ± 2.4 adducts/10^8 nucleotides, suggesting a relatively short persistence of the DNA damage induced by PAH in human PBL.

Detection of PAH-DNA Adducts in PBL of Psoriatic Patients by the ^{32}P-Postlabeling Method

The ^{32}P-postlabeling method is perhaps at present the most widely used technique for monitoring the human exposure to PAH. We quantified PAH-DNA adducts by the ^{32}P-postlabeling technique using both nuclease P1 enrichment and the butanol extraction procedures. Figure 1B shows the levels of total- and anti-BaPDE-DNA adducts in the 26 PBL samples obtained from the nine psoriatic patients and in the PBL samples from the 8 healthy subjects, using the nuclease P1 enrichment procedure. No statistically significant difference was found among the mean total DNA-adduct level before clinical therapy with TP (0.46 ± 0.18 adducts/10^8 nucleotides), after 8 days of continuos TP application (0.54 ± 0.36 adducts/10^8 nucleotides), and 16 days after the end of TP treatment (0.53 ± 0.26 adducts/10^8 nucleotides). Moreover, in healthy subjects the mean level of adducts (0.32 ± 0.24 adducts/10^8 nucleotides) was not statistically different from the mean levels of adducts in psoriatic patients. The autoradiograms of DNA adducts in the analyzed PBL samples revealed the presence of radioactive spots mainly in a distinct diagonal radioactive zone (DRZ) which is typical for aromatic DNA adducts (*15,16*). The same DRZ was found in ^{32}P-postlabeling digests of DNA from human skin mantained in short-term organ culture after treatment with CT-ointments (*17*), in DNA from PBL and from lung cells of lung cancer patients (*16*), and was shown to consist of a multitude of DNA adducts containing aromatic hydrophobic moieties, as indicated by their chromatographic behaviour (*15*). It must be noticed that only in seven samples of DNA from psoriatic patients we were able to detect the specific spot corresponding to the anti-BaPDE-DNA adduct.

Figure 1C shows the levels of total-DNA adducts in the 18 PBL samples obtained from the nine psoriatic patients using the butanol extraction procedure. Also, in this case no statistically significant difference was found among the mean total DNA adduct level before clinical therapy with TP (0.74 ± 0.38 adducts/10^8 nucleotides), after 8 days of continuous TP application (0.68 ± 0.25 adducts/10^8 nucleotides) and 16 days after the end of TP treatment (0.79 ± 0.25 adducts/10^8 nucleotides).

Moreover, although the comparison between the mean total DNA-adduct levels detected by the nuclease P1 method (0.58 ± 0.31 adducts/10^8 nucleotides) and that detected by the butanol method (0.73 ± 0.30 adducts/10^8 nucleotides) did not reveal any significant difference, the autoradiograms of 18 DNA samples obtained by the butanol procedure revealed the presence of additional radioactive spots not detected by the nuclease P1 method.

Comparison between the Level of Aromatic-DNA Adducts Detected by the ELISA Method and the ^{32}P-postlabeling Technique in the PBL from Psoriatic Patients

It must be noticed that the mean BaPDE-DNA-adduct level we detected by the ELISA method in the PBL of psoriatic patients is about 20 to 100 times higher than that we found by the ^{32}P-postlabeling technique on the same cell system. Other authors (*18,19*), using ELISA methods, found in the DNA from lung tissues of lung cancer patients BaPDE-DNA levels 5 to 10 times higher than those determined by the ^{32}P-postlabeling technique. In other studies, the postlabeling values of DNA adducts were significantly (10–100 fold) lower than those obtained by ELISA (*20*). The discrepancy between the results obtained by the two methods can be explained by cross reactivity of the antiserum against different DNA adducts, which enhances the responsiveness of the ELISA method as opposed to structurally related PAH adducts (*19*). The underestimation observed with postlabeling may also be due to some intrinsic proprieties such as poor efficiency of phosphorylation (*20*). Moreover, the nuclease P1 digestion of DNA, which is used in the present as well as in other studies to enhance the sensitivity of the ^{32}P-postlabeling assay, may result in dephosphorylation of certain adducts, impeding their ^{32}P-postlabeling, and may give an underestimation of the actual adduct level (*20*).

Benzo[*a*]pyrene Metabolism and DNA Adduct Formation in Cultured PBL of Psoriatic Patients

The topical application of CT solution to neonatal rats was shown to induce the aryl hydrocarbons hydroxylases AHH of skin and liver (*21*). The AHH in the skin of psoriatic patients have a lower activity and inducibility than in control subjects (*22*). Therefore, we evaluated the possibility that the treatment with CT influences the BaP activation and the binding of its metabolites to DNA. PBL samples, from five healthy subjects and 10 psoriatic patients, were analyzed after treatment *in vitro* with 2 μM (-)-7,8-dihydrodiol for 24 hr. The metabolism of (-)-7,8-dihydrodiol to syn- and antistereoisomers of BaPDE was determined by monitoring the formation of their corresponding tetrols: BaP-7,10/8,9 and BaP-7/8,9,10 for (+)-anti-BaPDE, and BaP-7,9/8,10 and BaP-7,9,10/8 for (-)-syn-BaPDE. As shown in Figure 2, anti-tetrols were the predominant isomers in all the

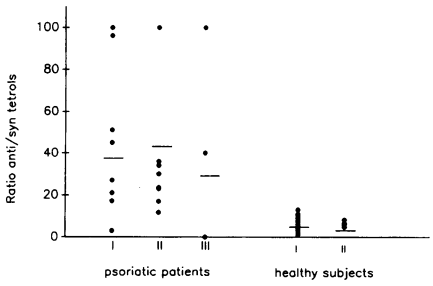

Figure 2. Ratio of anti/syn BaP-tetrols, formed *in vitro* by PBL of psoriatic patients and healthy subjects after treatment with (-)-BaP-7,8-dihydrodiol, and detected by HPLC. PBL were obtained from psoriatic patients before (I) or 4 to 10 days after the end of CT therapy (II), and from healthy subjects reanalyzed 2 to 3 times at distance of 10 days from one another (I, II).

examined PBL samples (mean ratio anti-/syn-tetrols: 23.2 ± 10.83), but significant levels of syn-tetrols, which clearly derived from the hydrolysis of (-)-syn-BaPDE, were also present. The mean ratio of anti-/syn-tetrols in healthy subjects (5.7 ± 0.35) was significantly lower than in psoriatic patients (32.1 ± 15.25) ($p<0.001$), whereas statistical comparison of this ratio in psoriatic patients, before and after treatment with CT, did not reveal any significant difference.

The analysis by HPLC of the DNA adducts, after incubation of lymphocytes with unlabeled 2 μM (-)-BaP-7,8-dihydrodiol for 24 hr (*13*), showed the formation of consistent amounts of (-)-syn-BaPDE-DNA adducts in the 15 PBL samples both from healthy subjects and psoriatic patients. The mean ratio of anti-BaPDE-DNA/syn-BaPDE-DNA adducts was 4.01 ± 3.27 pmol/μgDNA, and no significant difference in that ratio was found either between healthy subjects and psoriatic patients, or between psoriatic patients, before and after CT treatment (*13*).

Conclusion

The purpose of our study was to evaluate the genotoxic risk involved in the exposure of psoriatic patients to PAH by the detection of PAH-DNA adducts in their DNA isolated from PBL. Our data demonstrate no correlation between the levels of PAH-DNA adducts and the exposure to CT. With the lack of correlation between CT therapy and incidence of skin cancer in psoriatic patients (*4*), such data suggest that CT treatment of psoriasis should not be considered a potential genetic and carcinogenic risk for psoriatic patients. However, it must be notice that the DNA-adduct levels in nontarget tissues, like PBL, could not reflect the level of adducts in skin and lung cells, both of which have been shown to be the target tissues for DNA in human PAH exposures (*14,23,24*). Therefore, the lack of elevated DNA adducts in PBL, although representing a relevant information related to biomonitoring, does not allow to directly predict the skin cancer risk involved in CT therapies.

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